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In Antibody-Directed Enzyme-Prodrug Therapy (ADEPT), antibody - enzyme constructs localize to tumor tissue the toxification of non-toxic prodrugs. Recombinant fusion proteins are expected to overcome the limitations of chemical conjugates.

We have constructed fusion proteins of antibodies against the tumor antigens A33, Lewis-Y and fibroblast associated protein (FAP) with cytosine deaminase (CD), which converts 5-fluorocytosine (5-FC) into cytotoxic 5-fluorouracil (5-FU). Initially using a T7 polymerase-based expression system, plasmid vectors had been designed for the cloning and expression of the fusion constructs in bacterial and Pichia pastoris expression systems.

After initial production in E. coli, fusion proteins were produced in *P. pastoris* and purified from media supernatant with higher yield than from the previously used bacterial expression system.

During the reporting period, work on this project was limited due to the ongoing transfer of funds from to the primary investigator's new institution in Germany. However, the expression systems used could be further optimized to achieve good fusion construct yields with pilot proteins. With funds now available, the project is well set to continue successfully with the plannend animal experiments in the revised time frame.

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FOREWORD

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Prodrug Therapy for Breast Cancer Targeted by Single-Chain Antibodies F19 and 3S193

Introduction

Antibody-directed enzyme-prodrug therapy (ADEPT) employs antibody-enzyme constructs to achieve tumor-targeted activation of nontoxic prodrugs. In this project, recombinant fusion proteins of single-chain antibody fragments (scFv) and prodrug-activating enzymes are being constructed and evaluated based on the antibodies F19 and 3S193. During the first 14 months, it was planned to establish the cloning and expression strategy (Task 1), followed by the validation of the proposed ADEPT concept in vitro (Task 2). As had been previously reported, task 1 has met with considerable difficulties in establishing the protein production, which required repeated redesigning of the cloning and expression strategy. Recently, we have successfully transferred the previous expression system to the eucaryotic Pichia pastoris system. This system still required optimization, which has now been successfully completed with reproducible high yields and stable protein products. In addition, there have been indications that cytosine deaminase from Sacharomyces cerevisiae could provide higher activity and be better suited for expression in the yeast system than the bacterial isoenzyme. We have therefore cloned this enzyme and inserted it into the current expression system.

In autumn 2000, the principal investigator of this project moved to the Universitätsklinikum Benjamin Franklin of the Freie Universität Berlin, Germany, with the intention of continuing this project in collaboration with Memorial Sloan-Kettering Cancer Center, the original host institution. As previously reported, this move led to a more prolonged disruption of the work than anticipated. Initially it had been possible to resume laboratory work on this project successfully, with good progress to report on in 2001. While the best efforts of all parties involved were taken, the transfer of funds to Germany took longer than anticipated, and only in April 2002 have funds from this grant become available again for the principal investigator. To bridge this financing gap, the new host institution, the Universitätsklinikum Benjamin Franklin of the Freie Universität Berlin, employed the principal investigator full time as a clinical physician. This left no official work time for the pursuit of this project for most of the reporting period. Still, outside his work time the principal investigator continued to pursue this project by addressing the experimental issues mentioned above.

Report Body

As stated in the introduction, due to a complete interruption in funding, only very limited work was possible on this project in the past reporting period. Apart from administrative tasks, this was focused on further optimization of the Pichia pastoris expression system for the antibody-enzyme fusion proteins.

The funds have now been successfully transferred to the new host organization in Germany, the Universitätsklinikum Benjamin Franklin of the Freie Universität Berlin, with an extended time frame. This means that the funding period has been extended by one year, to compensate for the elapsed time without funding. Adminsitrative tasks such as the approval of the planned animal experiments were completed successfully, paving the way for a timely continuation of this project under the revised schedule.

In this context, it may be necessary to comment on a political discussion in Berlin that has been reported on in international science news. The newly elected Senate of Berlin had announced plans to close down the Universitätsklinikum Benjamin Franklin as a university hospital, meaning that research could no longer be conducted here. For the time being, realization of this plan has been averted. An expert commission is now evaluating concepts for the restructuring of the university medicine in Berlin at large, and the closing down of a single site is no longer the focus of the discussion. In any event, such a decision would be effective no sooner than in 2005. Thus, the immediate completion of the project funded by this grant is not jeopardized by this political development. Still, while this grant is a direct one to the principal investigator, indirect negative repercussions such as difficulties in finding personnel and other funding cannot be precluded for the future. The principal investigator regards it has his task of honesty to point out this fact to the funding organisation. In any case, however, he will be able to guarantee that the granted funds will only be used for the purposes of this project as described in the original proposal or modifications thereof that are dictated by the progress of the scientific work, and that he will be able to return any unused funds in the case that unforeseen developments should hinder the continuation of this project.

Expression of recombinant fusion proteins in Pichia pastoris

As detailed in the previous report, Pichia pastoris has long been described as giving high yields in the expression of foreign, recombinantly inserted genes as soluble proteins. After initial success with a bacterial expression system, we had found that this gave too little yields for in vivo testing of the produced recombinant proteins, and we thus switched to the Pichia pastoris system already in the previous year. In the past reporting period, a considerable proportion of the time yet available was dedicated to further optimization of this expression system. Continuing to use 1-liter shake flask cultures, induction and culture conditions were further optimized, and the resulting proteins from

supernatant tested for function and stability. With green fluorescent protein fusion constructs, we could achieve yields of several milligrams per liter culture, and the crude supernatant showed sufficient function for identification of antigen-positive cells in fluorescence microscopy and fluorescence flow cytometry (FACScan). This as well as chromatography purified material turned out to be very stable. After more than three months at –20°C, no sognificant loss in antibody binding or fluorescence activity was noticed. Furthermore, material stored at ambient temperature under various conditions for more than a week was still active for both qualities in fluorescence cytometry and showed high affinity in a Biosensor assay.

Yeast vs. bacterial cytosine deaminase

In the original grant proposal, cytosine deaminase from yeast has been selected for the 5-FC-converting enzyme because of the wide experience in its application in gene therapy and its ready availability. Cytodine deaminase from yeast, however, has been reported to be of higher specific activity. When the expression of scFv-construcs with cytosine deaminase from E. coli turned out to give much lower yields than scFv-GFP constructs, the yeast isoenzyme looked even more attractive, as due to different codon usage the eucaryotic protein might be better expressed in yeast than the bacterial one. Finally, a recently published report on the ultrastructure of bacterial cytosine deaminase identified it as a hexamer. While its monomers are expected to be enzymatically active, the yeast isoenzyme, which is naturally a dimer, may reach higher activity in fusion constructs.

We therefore cloned the yeast cytosine deaminase from S. cerevisiae, using flanking primers based on the published sequence, and inserted into the scFv-fusion construct vector frames. Expression of these constructs is currently underway, and a final assessment of their advantages in production and function (or lack thereof) can not yet be given.

Educational aims and Summary

During this reporting period, no funds from this grant were actually available. Hence work on this project could not continue as planned, but had to be halted while the principal investigator was funded directly from his new institution in a clinical position. However, with the joint effort of all parties involved, the transfer of funds has now been gratefully completed.

Nevertheless, despite the complete interruption of funding, the expression system could be further optimized and extended, and a robust and versatile expression system yielding highly stable material is now available. This time was also used to start improving the complete prodrug system by replacing bacterial cytosine deaminase with the isoenzyme from yeast.

Thus, remaining obstacles from the previous tasks of this project have been addressed, and the formal requirements for the planned animal experiments have been met. The forced interruption of this project has thus been well used to prepare for its continuation in the revised time frame.

Appendix

Key Research Accomplishments

- Technical improvement of the Pichia pastoris expression system and in vivo testing of the produced proteins for function and stability

- Cloning and expression of fungal cytosine deaminase as a fusion partner for the selected single-chain antibodies

Reportable Outcomes

Due to the described interruption in funding and work, no reportable outcomes could be generated during this reporting period